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EXPERIMENTAL STUDIES ON AIR-BORNE INFLUENZA INFECTION AND THE DISINFECTION OF AIR

- COMMUNIST CHINA -

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EXPERIMENTAL STUDIES ON AIR-BORNE INFLUENZA INFECTION AND THE DISINFECTION OF AIR

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I. The Construction and Operation of the Apparatus

Infection via the respiratory tract is by no means less prevalent or less severe than infections via other routes. It often endangers the people's labor capacity and health. Therefore, it is essential in preventative medical practice to study the different aspects of respiratory infection, including the mechanism of air-borne infection and its disinfection. There are numerous reports and reviews concerning these studies in other countries, especially on the problem of disinfection. However, such study in China is still very limited.

In experiments dealing with respiratory infection, the experimental animals are infected through the contaminated air. The pathogen-containing suspension is sprayed from an atomizer into the air to form very fine droplets (aerosol), which are inhaled by the animals during their natural breathing. Since this aerosol may also infect human beings, it is necessary to safeguard laboratory personnel during the experimental procedures, and all apparatus must be properly sterilized. It is necessary to control precisely the experimental conditions and to carry out the experiments in a definite environment, so that we may interpret the findings more accurately. Rosebury has designed an apparatus with a certain degree of safety and some other merits, but it also has the following shortcomings:

1. It is too complicated in structure, and takes four to five to operate.

2. There is no thermostatic control for the cloudy chamber. In consequence, the temperature inside the cloudy chamber is easily affected by the room temperature and thus causing a change of humidity in the chamber.

3. Since there is only one air-lock, it is rather inconvenient in carrying out experiments at different intervals.

4. The cloudy chamber is a horizontal cylinder, within which the aerosol is sprayed in a direction parallel to its longitudinal axis. Even in motion, these droplets may precipitate, and result in a variation of the aerosol concentration in the air.

Druett and May have designed an apparatus which is similar to a wind pipe. The cloudy chamber is a vertical cylinder, within which the aerosol moves from top to bottom, thus circumventing the problem of concentration variation of aerosol in the air as previously mentioned. However, its structure is very complicated, and not suitable for general laboratory use. Besides, its cloudy chamber is rather small (for overcoming the shortcoming mentioned above), and it is not suitable for the tests of chemical disinfection. The other apparatus designs are those of Weiss & Segeler, Middlebrook, Leif & Krueger.

Since 1954, we have improved the Rosebury apparatus. Now we have an apparatus which is just as safe, but simpler in structure, and devoid of the above-mentioned shortcomings. We call it the air-borne infection cabinet. Its complete structure and operation are discussed as follows:

A. The Complete Set-Up of the Air-borne Infection Cabinet

The air-borne infection cabinet consists of a cloudy chamber, a control unit, and ancillary equipment.

1. The Cloudy Chamber.

The cloudy chamber is shown in Plates 1, 2, and 3. It is essentially a cylinder with a double wall made from the steel plates, 0.635 cm in thickness. The cylinder measures 59 cm in its inner diameter, 100 cm in length, and 273 liters in capacity. Its inner and outer walls are 2.5 cm apart,

forming a water jacket. A tightly-closed door is bolted in the front of the cloudy chamber.

On the left side of the chamber, there are two windows, 16 cm in diameter; to them are attached a pair of rubber gloves, 65 cm in length. The gloves form a 30° angle with the bottom of the chamber for the convenience of manipulation.

On the right side of the chamber, there are two small cylindrical antra, the airlocks, measuring 18 liters. There are two tightly-closed doors at both ends of each airlock, one leading to the cloudy chamber and the other opening to the exterior. These airlocks facilitate the transfer of the experimental animals at different intervals.

On the top of the chamber, there is an observation window, 16 cm in diameter, which has a pane of pyrex glass, 1.6 cm in thickness. There are also a pressure gauge, a pressure control, a thermometer, a hygrometer, and a lamp on the top of the chamber.

In the rear of the chamber, there are three air pressure pipes. The cloudy chamber and the air locks communicate through the steel pipes with the high pressure boiler, the pumps, the air filter, and the drain.

2. The Control Unit.

It consists of the following parts (Plate 4):

(1) The aircompresser is converted from a high pressure rotatory pump. The pressure is adjustable. When the pressure is one kg per square cm, the gaseous flow rate will be 30 liters per minute. The air passes through a filter made of four to five layers of fine gauze thinly greased with vaseline. It then goes into the rotatory pump to be compressed and is directly conducted to the air cooler.

(2) The air temperature and humidity control consists of a cooler, a drier, a heater, and a humidifier.

The cooler is a brass spiral condenser and is bathed in a cooling solution. The humidifier and the drier are two metal cylinders, 16 cm in diameter, and 22 cm in height, containing either a solution or a drying agent. The heater is a 150-watt electrical device.

The compressed air, after being cooled, is again passed through the filter, and then separately comducted into the drier, the heater, or the humidifier. Finally it enters into the cloudy chamber through the flow-meter.

(3) The water jacket thermostat.

The temperature of water inside the double walls is controlled by two one-kilowatt electrical heaters separately located at each end of the bottom of the jacket. When the water bath is maintained at 30°C and the room temperature at 12°C, the change of water temperature is within 1°C, and there is no appreciable change of temperature in the cloudy chamber. This indicates the important role played by the thermostat in maintaining a constant temperature when the difference between the chamber temperature and the room temperature is above 15°C.

(4) The exhaustion device is an ordinary rotatory pump. Under normal atmospheric pressure, the exhaustion flow is 20 liters per minute. It serves to expel the air within the cloudy chamber and airlocks out of the building.

The complete set-up is shown in Plate 6.

3. Ancillary Equipment.

(1) The incinerator is a high temperature electrical furnace. A tubular incinerating apparatus is formed from the thermoelectrical wire in the furnace. Between this apparatus and the insulating shell, there is a bell-shaped partition, forming a winding channel. The temperature inside the furnace is around 800°C. The contaminated air from the exhaustion device is passed into the double wall of the incinerator, around the center of the furnace, and then out of the building. We found that no bacteria could be cultured from 10 ml of sterile broth after it had been exposed to 150-300 liters of incinerated air at a flow-rate of 30 liters per minute, whereas the non-incinerated air gave a positive result.

(2) The Atomizer. An atomizer is indispensable in the air-borne infection experiments. Boyland, et al., have reported that droplets of a diameter of one micron can reach the pulmonary alveoli at any respiratory rate, and be absorbed there, whereas droplets above five micra or below one micron in diameter fail to do so. Sonkin has reported

that droplets with a diameter as large as 12 micra cannot reach the alveoli; thus they can only induce an upper respiratory infection. For this reason, we have to use an atomizer which produces droplets of a diameter less than 12 micra. We have made a vertical atomizer (Plate 5), which is a combination of Deoilbiss #40 and Chicago atomizers. This atomizer is operated on compressed air at 14-15 pounds per square inch. When 1 percent to 0.1 percent rat lung suspension in 0.1 M phosphate buffer is sprayed by this apparatus, 95 percent of the droplets, as determined by May's method are 1-5 micra in diameter.

(3) The animal transfer box, shown in Plate 1, is used for a safe transfer of the experimental animals from the cloudy chamber, via the airlock, into the feeding cabinets. If the pathogens used for the experiment are highly infectious or dangerous, the animals may be kept in the transfer boxes for feeding and observation. The upper and lower sides of the transfer box can be connected to the ventilator through metal pipes.

(4) The animal feeding cabinet is a simple ventilating cabinet with a door for communication with the animal transfer box, and it is also equipped with a pair of gloves for manipulation.

(5) The infected animal cages are small cages made from one-mm mesh wire gauze.

B. Operations of the Apparatus

The operation of this apparatus consists of safety check, preparation and adjustment, experimental procedure, and disinfection.

1. Safety Check.

The purpose of the safety check is to prevent infection of the laboratory personnel during the experiments. Before the start of each experiment, the compressed air is blown into the cloudy chamber, the airlocks, and the pipes until the air pressure reaches one kg per sq cm. Then the apparatus is kept under close observation for 18 hours, and it must be ensured that the drop of pressure inside the apparatus will not exceed 0.1 kg during the period of observation. If the decrease of pressure exceeds this limit, the leakage

must be detected by applying a saturated soft soap solution to all joints of the apparatus, which must be repaired.

2. Preparation and Adjustment

Adjust the temperature of the water jacket before starting the experiment. Allow no liquid inside the cloudy chamber. Start the air compresser and the exhaustion pump, and adjust the rate of air supply at 30 liters per minute, or at any rate that is required by the experiment. Adjust the ratio of air passing through the drier, the heater, and the humidifier in order to maintain a certain temperature and humidity of the air. The air, after being so treated, may or may not be combined before entering the atomizer. Adjust the rate of exhaustion at the same time, so that the air pressure inside the cloudy chamber is about two to three cm of H_2O below atmospheric pressure. When all adjustments are properly completed, allow the apparatus to run for several minutes, then turn off the compresser and air supplier, and be ready for the experiments.

3. Experimental Procedure

Transfer the pathogen suspension of known concentration into the atomizer. Tightly close the inner door of the airlock, and simultaneously start the air compresser and the exhaustion pump. Observe and record the total time of spraying, the flow rate of air as registered by the flow meters, and the temperature and humidity of the cloudy chamber before and after spraying. We have the following equation for the calculation of the pathogen density, D_o, in the air blown into the chamber.

$$D_o = ad\left(1 - \frac{1}{e^n}\right) \quad \text{..... (1)}$$

a = Efficiency of the atomizer

$$= \frac{\text{The actual number of living pathogens per liter of air in the chamber}}{\text{The calculated number of living pathogens/liter of air in the chamber}}$$

d = density of pathogens in the air of cloudy chamber = $\frac{CV}{W}$

C = Concentration of pathogens in the suspension, LD_{50}/ml, or Number of living bacteria/ml in the case of bacterial infection.

V = Rate of spraying of suspension, ml/min.

W = Rate of air flow into the chamber, liter/min.

n = Turnover rate of air inside the chamber

= $\frac{\text{Total amount of air blown into the chamber}}{\text{Capacity of the cloudy chamber}} = \frac{WT}{v}$

T = Total time of spraying, min.

v = Capacity of the cloudy chamber, liter.

e = 2.7183 = constant.

Formula (1) is converted into

$$D_o = a \frac{CV}{W} \left(1 - \frac{1}{2.7183 \times \frac{WT}{v}} \right) \quad \ldots\ldots\ldots \quad (2)$$

Unit is LD_{50}/liter.

Theoretically, an accurate density of pathogens in the air can be derived from this equation, provided the air is constantly in motion and agitated, and the aerosol will not condense on the walls of the chabmer due to a high relative humidity. However, when the air is static, the aerosol precipitates and condenses, thus its concentration decreases as time elapses. Therefore, a correction has to be made on the basis of the experimental findings.

Furthermore, there is a decrease of the number of living pathogens due to dehydration, oxidation, and spontaneous death of the pathogens when they remain in the stagnant air. In a quantitative determination, this factor has to be taken into account and a correction should be made in terms of time. According to Lidwell, et al., the density of

pathogens in the air at t time after the cessation of spraying is as follows:

$$D_t = D_o e^{-(R+S)t}$$

e = the base of the natural logarithm = 2.3026

R = the turnover rate of air inside the cloudy chamber.

S = the diminishing constant of pathogens in static air.

In static air, another correction that should be added is the dilution of the pathogen density due to mixture of the air in the cloudy chamber and that of the airlock during the transfer of the experimental animals. This correction may be disregarded if the density of pathogens in the air is very high. However, it should be made according to the following formula if the density is low.

$$D' = D_t \frac{V}{V+U} \quad \text{..... (3)}$$

D' = the density of pathogens in the air inside cloudy chamber.

U = the capacity of the airlock.

V = the capacity of the cloudy chamber.

D_t = the actual density of pathogens in the air of the cloudy chamber at t time (before the animal transfer).

The experiments may be carried out during the spraying of the pathogen suspension, or at a certain time after the spraying while the air is either in a constant motion or static, as is related to the purpose of the particular experiment. This apparatus may be utilized for the following studies:

(1) The determination of the minimal dose of respiratory pathogens required for an effective air-borne infection of

the animals, and the mechanism of infection and immunization of the animals as induced by the air-borne pathogens.

(2) The effect of the size of droplets upon the virulence and endurance of pathogens in the air.

(3) The effect of air temperature and humidity upon the the activity of pathogens.

(4) The effect of physical and chemical agents upon the activity of air-borne pathogens.

(5) The effect of the non-specific change of aerosol in the air and physical environment upon the susceptibility of animals to the respiratory pathogens.

(6) The effect of the presence of two different pathogens in the air upon the infection of animals.

4. Disinfection

At the end of the experiment, the entire apparatus may be sterilzed by (a) high-pressure steam, (b) flowing steam, and (c) chemical fume or vapor.

Conclusion

An apparatus which can be safely used for the study of air-borne infection and air decontamination is reported, and its operation and application are discussed.

II. The Effect of Relative Humidity and Temperature upon the Virulence and Endurance of Air-borne Viruses

In the study of air-borne infection of pathogenic microbes, it is necessary to undetstand the effect of relative humidity and temperature upon their virulence and endurance. DeOme reported that an increase of air temperature and relative humidity accelerates the death rate of air-borne S. pullorum. Dunklin and Puck reported that the survival period of pneumococcus and Staphylococcus albus, as sprayed into the air in

the form of their cultural mist, is shortest at a relative humidity of 45-60 percent. Lidwell and Lowbury have found that the death rate of Staphylococcus aureus and Streptococcus pyogenes is in a direct proportion to the relative humidity.

Loosli, et al., reported that the air-borne virus influenza A maintains a longer period of virulence toward white mice at a lower relative humidity. Lester reported that PR8 stock virus, when diluted in the heart broth and sprayed into the air, possesses the least virulence at a relative humidity of 45-60 percent, but no such effect of relative humidity can be observed after the virus suspension has been dialyzed. So far, we have not come across any reports concerning the effect of air temperature on air-borne viruses.

This article deals with the effect of relative humidity and temperature on the virulence of air-borne virus and their persistence of endurance.

Materials and Methods

1. Virus. FM_1 virus stock, grown in the rat lungs until the 16th generation, is inoculated inside the nostrils of white mice. Its LD_{50} titer is determined as $10^{-4.7}$.

2. Preparation of the virus suspension. The infected white mice are exsanguinated through resection of the carotids. The lungs are removed by a sterile process, and are washed in the steril normal saline to remove the blood. After being weighed, the lungs are transferred into a tissue homogenizer. At this point, a certain amount of phosphate buffer (1/15 M, pH 8.0, containing 1,000 units of penicillin and 500 units of streptomycin per ml) to make a 10 percent suspension. Homogenize and then Centrifuge the homogenate at 3,000 rpm for 30 minutes. Pipet the supernatant, titrate its lethal effect on the white mice, and calculate the LD_{50} titer by the Reed and Muench method. Place the supernatant in the small test tubes, and store them at -30°C in the refrigerator. It has to be used within four days after preparation.

3. Adjustment of air humidity and temperature inside

the cloudy chamber. Adjust the temperature of the water jacket of the cloudy chamber to keep the temperature within the chamber at either 15°±0.5°C or 25°±0.5°C. For the humidity adjustment, $ZnCl_2 \cdot 1\frac{1}{2} H_2O$, KNO_2 or the saturated solution of $(NH_4)_2SO_4$ is placed in the chamber in addition to the humidifier, in order to expedite the process. The relative humidity inside the chamber is maintained around 20 percent, 50 percent, and 85 percent. One should use a 0.25 ml tuberculin syringe to inject a few drops of distilled water on a small heated plate inside the chamber ot raise the relative humidity in the chamber to 20 percent, 55 percent, and 90 percent. Then the spraying of virus suspension is begun. The diameters of 95 percent of the droplets are within 1-5 micra, with an average of 3.5 micra.

Results

1. The Effect of Relative Humidity and Temperature on the Virulence of the Air-borne Influenza Virus. A certain aliquot of virus suspension is sprayed into the cloudy chamber. The air, after being used for atomizing the suspension, is withdrawn from the cloudy chamber and passed through an asbestos filter into the air compresser. It is then returned into the atomizer. Each liter of the air thus contains 0.0009 ml of virus suspension. By claculation, the concentration of virus in the air is 0.78 LD_{50} per liter. After the virus suspension has been sprayed into the chamber the air inside the chamber is constantly circulated by a small fan.

Five groups of healthy three-week-old white mice are then placed in the chamber (each group consisting of six animals in a special wire cage). These groups are removed individually from the chamber after $\frac{1}{2}$, 1, 2, 5, and 10 minutes of exposure there. The animals are then fed and observed for two weeks. The exposure time, in minutes, required to kill 50 percent of the animals is calculated, as well as the LD_{50} of virus inhaled by each animal under various conditions. The LD_{50} approximately equals the product of the calculated exposure time, the concentration of virus in the air, and the tidal volume of natural breathing of the animals. The tidal volume of each white mouse is determined by the method of Loosli, et al., to be 0.02 liter/min. The variation of tidal volume due to changes in temperature and humidity,

and the minute difference of virus concentration in the air, after it has been breathed by the animals, are generally disregarded in calculation.

The results of the experiments and calculation are presented in Table 1. It is shown that there is no apparent effect of temperature on the viral virulence between 15° and 25°C, whereas a certain effect is exerted by the relative humidity. When the relative humidity is 90 percent, the exposure time required to kill 50 percent of the animals is less than one half the exposure time for the same effect at a relative humidity of 20 percent.

Table 1
The Effect of Relative Humidity and Temperature on the Virulence of the Air-borne Influenza Virus

Temperature	Relative Humidity Before Virus Spraying	Relative Humidity After Virus Spraying	Exposure Time of White Mice (Minutes) ½	1	2	5	10	Exposure Time Required to Kill 50% of Animals (Minutes)	Amt of Virus Inhaled by Each Animal at 50% Death Rate(LD_{50})
15°±0.5°C	20%	24%	0/6*	1/6	3/6	4/6	5/6	2.48	0.039+
	55%	57%	2/6	2/6	3/6	6/6	6/6	1.41	0.022
	90%	91%	2/6	3/6	4/6	6/6	6/6	1.00	0.016
25°±0.5°C	20%	23%	0/6	2/6	2/6	4/6	6/6	2.75	0.043
	55%	56%	1/6	2/6	4/6	6/6	6/6	1.37	0.021
	90%	90.5%	3/6	3/6	4/6	6/6	6/6	0.92	0.014

*The denominator represents the number of infected animals, and the numerator the number of animals that died.
+Based on 0.016 LD_{50} per animal per minute.

2. The Effects of Relative Humidity and Temperature on the Persistence of Virulence of Air-borne Influenza Virus. The does of virus used in this experiment is the same as that in the previous experiment. However, the air in the chamber remains static with the fan off after the spraying. The groups of white mice are placed inside the chamber 5, 10, 20 and 30 minutes after the cessation of spraying. The animals are left there for 10 minutes to be allowed to breath a sufficient quantity of the infected air. They are then removed from the chamber to be fed and observed for two weeks. The maximal time in which the air-borne virus maintains its lethal effect toward 50 percent of the animals is calculated. This maximal time reflects the persistence of viral virulence.

The results of this experiment, as shown in Table 2, demonstrates that, within the temperature range used here, there is no appreciable effect of temperature on the virulence persistence of the air-borne virus. However, at a relative humidity of 20 percent, the viral virulence persists for 21.7-24.1 minutes; at a relative humidity of 55 percent, it persists for 17.5-18.4 minutes; at a relative humidity of 90 percent, it persists for 14.7-16.0 minutes. Thus, it appears that the virus lives a shorter period at a high relative humidity than at a low relative humidity. This point will be discussed later.

Table 2

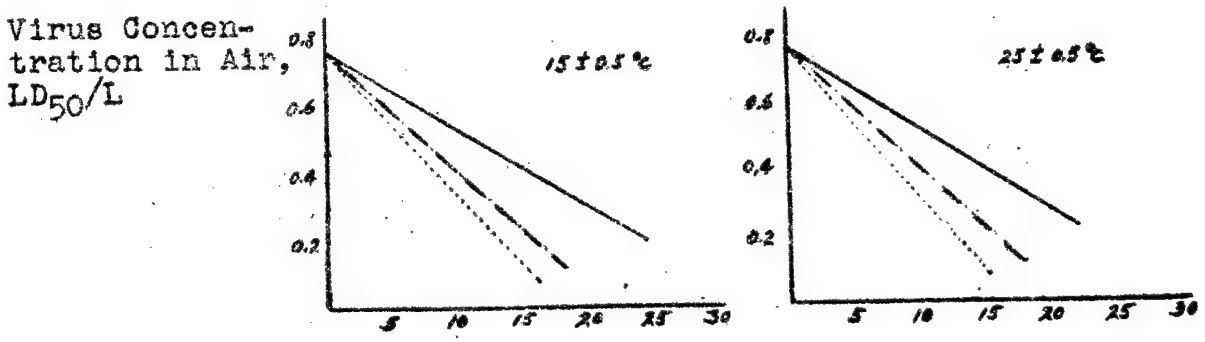
The Effect of Relative Humidity and Temperature on the Persistence of Virulence of the Air-Borne Influenza Virus

Temperature	Relative Humidity Before Virus Spraying	Relative Humidity After Virus Spraying	Time After Virus Spraying (minutes) 5	10	20	30	Max. Time Air-borne Virus Retains Lethal Effects on 50% of Animals (Minutes)	Calculated Final Viral Concentration in Air Lethal to 50% of Animals, (LD_{50})
15°±0.5°C	20%	23%	6/6*	4/6	4/6	3/6	24.1	0.20**
	55%	56.5%	6/6	4/6	3/6	1/6	18.4	0.11
	90%	90.5%	6/6	5/6	1/6	1/6	16.0	0.08
25°±0.5°C	20%	23%	6/6	4/6	3/6	3/6	21.7	0.22
	55%	56%	6/6	4/6	2/6	2/6	17.5	0.11
	90%	90.5%	6/6	4/6	1/6	1/6	14.7	0.07

*Same as in Table 1.
**Derived from the data in Table 1.

The concentration of virus in the air after spraying has been calculated to be 0.78 LD_{50}/l. However, this concentration may be changed at different relative humidities, since the virus retains its lethal effect on 50 percent of the animals for a longer period at a lower relative humidity than at a higher relative humidity. Therefore, the spontaneous diminishing rate of viral concentration in the air is more rapid at a higher relative humidity than at a lower relative humidity (Fig 1).

Fig. 1 Spontaneous Diminishing Rate of Viral Concentration in Air at Different Relative Humidities



———— 20-24% Relative Humidity

—.—.—.— 55-57% Relative Humidity

..... 90-91% Relative Humidity

Discussion

The first part of this experiment proves that there is a certain effect of the relative humidity of air upon the virulence of the influenza virus, FM_1 stock, and that the virulence of air-borne virus is directly proportional to the relative humidity.

According to Lester, the lowest virulence of virus at a relative humidity of 45-60 percent is probably due to the effect of sodium chloride in the broth medium, since the effect of relative humidity disappears after the viral suspensions have been dialyzed. He has no further explanation for this phenomenon. Loosli, et al., prepared a virus suspension with a broth containing 10 percent horse serum, and sprayed it in the air at different relative humidities. They found that the virulence of virus toward the white mice was entirely the same in all conditions. Since they did not determine the viral concentration in the air, it is possible that the effect of relative humidity may not be revealed when the viral concentration is too high.

Sonkin reported that the infection of the respiratory tract is related to the size of the microbe-carrying droplets. The droplets with a diameter below 1.8 micra may induce an infection of the lower respiratory tract, and those between 1.8 and 12 micra an infection of the upper respiratory tract.

Landahl and Herrmann reported that the probability of the droplets passing through the nostrils of animals is in direct proportion to their diameters, and, on the other hand, the probability of the droplets remaining inside the lungs is inversely proportional to their diameters. Their findings demonstrated that, within a certain range of diameters, these droplets reach a maximal percentage in passing through the nostrils and remaining in the lungs. Boyland, et al., found that this range of diameters is two to four micra.

Henderson reported in 1952 that the dehydration rate of the droplets in the air is inversely proportional to their diameters and the relative humidity; therefore, with a smaller diameter and at a higher relative humidity, the droplets will dry faster.

From the above findings, the pathogen-carrying droplets may be rapidly dehydrated at a very low relative humidity, and their diameters may thus be greatly reduced. Consequently, these shrunken droplets are unlikely to remain in the lungs and induce infection. This explanation supports our observations. However, it has to be pointed out that at a high concentration in the air, the virus may retain 100 percent of its virulence even at a very low relative humidity, since, under this condition, the animals often receive a greater amount of virus than the lethal dose.

The authors have also shown that the rate of growth rate of a small amount of virus (FM1) in the lungs of mice may be increased, when some hypotonic saline is used as a non-specific stimulus for the nasal inoculation of the animals. We have also found, in this same treatment, that the virus of chick amniotic fluid is rapidly adapted to the lungs of mice. Thus, a non-specific stimulation may increase the death rate of the infected white mice at a high relative humidity.

The second part of this experiment has shown that a certain relation exists between the relative humidity and the virulence persistence of the air-borne virus. Since the droplets are not rapidly dehydrated at a higher relative humidity, they are apt to precipitate or to condense on the wall; this results in a great decrease of viral concentration in the air. We have often observed many minute water drops on the wall and on the base of the chamber soon after the spraying of viral suspension at a high relative humidity. However, at a low relative humidity, the droplets dry very quickly and are reduced in volume; therefore, they are not inclined to precipitate or to condense on the wall so as to cause a great reduction of viral concentration in the air. As a result, it seems that the persistence of viral virulence in the air is inversely proportional to the relative humidity.

Loosli, et al., Robertson, et al., and Edward, et al., have found that the PR8 stock influenza virus loses its virulence within 60 minutes after it is sprayed in the air under normal conditions. Loosli, et al., believe that this loss of virulence by the air-borne virus at a high relative humidity is essentially due to the destruction of the microbe rather than to the precipitation of droplets.

However, Edward, et al., think that the precipitation of droplets plays an important role here. Merekalova has proved that M-12 stock influenza virus retained its virulence toward the white mice 3½-4½ hours after it had been sprayed into the air. Loosli, et al., also reported that PR8 stock virus killed 40 percent of the white mice 12 hours after it was sprayed in the air at a relative humidity of 17-24 percent.

It is hard to attribute the rapid loss of virulence by the air-borne virus to the death of the microbes alone. Certainly the precipitation and condensation of the droplets may exert a more important influence. It has to be pointed out that thereis a close relation between the virulence persistence of the air-borne virus and its concentration in the air. At a higher concentration, the virus retains its virulence for a longer period inspite of the precipitation and condensation of the droplets, and the destruction of the microbes. Loosli and Merekalova might have used a very large amount of virus in their experiments, and this may account for the long persistence of virulence of their virus.

This experiment has proved that the temperature has no apparent effect on the viral virility and its persistence.

It has also been demonstrated in this experiment that the infection by air requires a much smaller quantity of virus than the infection by nasal drip. If the amount of virus used for nasal drip infection to kill 50 percent of the white mice is 1 LD_{50}, the amount of virus required for the same effect by air infection is only 0.014-0.016 LD_{50} at 90 percent relative humidity, 0.021-0.022 LD_{50} at 55 percent relative humidity, and 0.038-0.043 LD_{50} at 20 percent relative humidity. These data have shown that the dose of virus effective for infection by air is only 1/70-1/23 of the dose necessary for a similar infection by nasal drip. This probably explains why the prevalence of influenza is so great.

Conclusions

The relative humidity has a certain influence upon the virulence of the FM_1 stock air-borne influenza virus. The viral virulence is greater, but its persistence is shorter at a higher relative humidity than at a lower relative humidity. These findings have been discussed.

Within the range of experimental temperature (15°-25°C), there is no apparent difference in the viral virulence and its persistence.

III. The Chemical Sterilization of the Air-borne Influenza Virus

Sterilization of the air in places used for group gatherings during periods of prevalence of a viral infection may achieve a certain effect of prophylaxis. Well, et al., have reported that during the prevalence of mumps and measles, a sterilization of the classrooms of the grade schools by ultraviolet rays greatly reduces the incidences of these infectious among the students. Stokes and Henle reported that ultraviolet rays and propylene glycol have a definite value in the prophylaxis of influenza. Many other workers have used sodium or calcium hypochlorite solution, and the fumes or vapor of triethylene glycol and propylene glycol in their studies of air decontamination in regard to influenza.

Vashkov reported the use of bleaching powder solution for the sterilization of the air-borne influenza virus. Tsui-pu-li-ssu-k'a-ya has pointed out the importance of bleaching powder solution in the disinfection and prevention of influenza. However, Chang et al., have found that sodium hypochlorite and bleaching powder solution have in the disinfection of the air-borne influenza virus. Instead, they believe that lactic acid is the most effective agent.

Vashkov has suggested the use of alcohols and glycerols for disinfection. Since there is still a lack of evidence in regard to the value of these chemicals in disinfecting air-borne influenza, this experiment will deal with the

effect of the aerosols of calcium hypochlorite, lactic acid, formalin, ethanol, or glycerol in disinfection.

Materials and Methods

1. Virus. The FM_1 stock of influenza virus is grown in the lungs of white mice by nasal inoculation until the 17th generation or further. The LD_{50} titer of virus regarding the white mice is 10^{-5} or greater.

2. Preparation of the Virus Suspension. Inoculate each of the three-week old normal white mice with 0.03 ml of 0.1 percent suspension of lungs from the infected white mice. Three days after the inoculation, remove the lungs from the animals by a sterile process, and make a 10 percent suspension of these tissue with a 1/15 M phosphate buffer at pH8.0. Centrifuge this suspension at 3,000 rpm for 30 minutes. Pipet the supernatant, and determine its LD_{50} titers regarding the white mice by the nasal inoculation method. Use a 1 percent suspension for spraying in each experiment, and for each liter of air spray 0.00412 ml of virus suspension. Since the virus preparation used in each experiment has a different LD_{50} titer, the amount of virus sprayed into the chamber varies. However, this variation does not exceed fivefold.

3. Adjustment of Temperature and Humidity of the Cloudy Chamber. Follow the same procedure described in I and II. The temperature of the cloudy chamber is set at $15^{\circ}\pm0.5^{\circ}C$, and the relative humidity at 50 percent-55 percent.

4. Chemical Disinfectants

(1) Clacium Hypochlorite. The commercial purified bleaching powder, which has been analyzed to contain 69 percent active chlorine, is made into 0.1 percent, 0.2 percent, and 0.5 percent solutions before use.

(2) Lactic Acid. Use purified reagent, containing 84 percent lactic acid. Dilute to 1/21, 1/42, 1/84, and 1/210 before the experiment.

(3) Formalin. Use formalin containing 36 percent formaldehyde. Prepare 3 percent, 6 percent, and 12 percent solutions before the experiment.

(4) Ethanol. Use 9 percent alcohol. Prepare 10 percent and 20 percent solutions before the experiment.

(5) Glycerol. Use chemically prue neutral glycerol. Dilute to 4 percent and 8 percent before the experiment.

5. Determination of Disinfective Efficiency of the Chemicals. After the temperature and relative humidity of the cloudy chamber have been adjusted in the manner previously described, 0.001 ml of 10 percent virus suspension is sprayed into the chamber, and this is followed immediately by 0.005 ml of chemicals at different concentrations sprayed into the chamber from a Devilbiss atomizer. The air inside the chamber is well mixed by a small fan for five minutes. The groups of three-week-old normal white mice, 12 animals in each group, are transferred one at a time into the chamber 5, 10, 20, and 30 minutes after the spraying of chemicals. The animals are left in the chamber for 10 minutes, and then are removed to be fed and observed for two weeks. The disinfective efficiency of the chemicals is calculated according to the following formula:

$$\text{Disinfective efficiency} = \frac{\text{No of dead controls} - \text{No of dead experimentals}}{\text{Number of dead controls}} \times 100.$$

Results

1. Disinfection with Calcium Hypochlorite. As shown in Table 3, at a concentration of 0.005 ml per liter of air, the 0.1 percent solution of calcium hypochlorite containing 0.0017 mg chlorine, reaches a disinfective efficiency of 100 percent in 30 minutes, and the 0.2 percent solution, containing 0.0035 mg chlorine, reaches a disinfective efficiency of 83.4 percent in 5 minutes, and that of 100 percent in 10 minutes.

The disinfective efficiency obtained by scrubbing the chamber floor with a 0.2 percent solution of bleaching powder is about the same as that achieved by spraying 0.1 percent solution into the chamber, and the disinfective efficiency by scrubbing the chamber floor with a 0.5 percent

solution is equivalent to that by spraying with 0.2 percent solution. In fact, the scrubbing method uses a much larger amount of chemical solution than the spraying method.

2. Disinfection with Lactic Acid. As shown in Table 4, there is a limited disinfective effect of lactic acid, when it is sprayed into the air in the form of a dilution of 1/210-1/42, containing 84 percent pure lactic acid, or at a concentration of 0.02-0.1 mg lactic acid per liter of air. However, if a lactic acid solution of 1/21 dilution is used and its concentration in the air is 0.2 mg per liter, its disinfective efficiency reaches 100 percent in 30 minutes.

3. Disinfection with Formalin. As shown in Table 5, formalin has a definite disinfective effect of the air-borne influenza virus. A 100 percent disinfective efficiency can be achieved in 30 minutes at a concentration of 0.05-0.1 mg formaldehyde per liter of air, or in 20 minutes at 0.2 mg formaldehyde per liter of air.

4. Disinfection with Ethanol and Glycerol. As shown in Table 6, there is a questionable disinfective value of ethanol at 0.4-0.8 mg per liter of air, and glycerol seems to have a deleterious rather than a disinfective effect, since the death rate of the animals is increased after its use.

[Tables follows]

Table 3

The Disinfective Effect of Calcium Hypochlorite (Purified Bleaching Powder) on the Air-borne Influenza Virus

Virus		Chemical Disinfectants					No of Animals died/Number infected		
LD_{50} Titer	Calculated Viral Concentration in the Air After Spraying	Concentration	Methods of Application	Amount Used	Calculated Viral Concentration in Air After Spraying	Reaction Time, Min.	Experimentals	Controls	Disinfective Efficiency, (%)
$10^{-6.2}$	25.0 LD_{50}/L	0.1%	Spraying	0.005ml per L of air	Equivalent to 1.7 x 10^{-3} mg chlorine/L	5	8/12	12/12	33.3
						10	5/12	12/12	58.3
						20	3/12	10/12	70.0
						30	0/12	6/12	100.0
$10^{-5.5}$	4.98 LD_{50}/L	0.2%	Spraying	0.005 ml per liter of air	Equivalent to 3.4 x 10^{-3} mg Chlorine/L	5	2/12	12/12	83.4
						10	0/12	12/12	100.0
						20	0/12	8/12	100.0
						30	0/12	4/12	100.0
$10^{-5.6}$	6.32 LD_{50}/L	0.2%	Scrubbing the Chamber Floor	90 ml/ sq meter	--	5	5/12	12/12	58.2
						10	2/12	12/12	83.4
						20	1/12	8/12	87.7
						30	0/12	5/12	100.0
$10^{-5.6}$	6.32LD_{50}/L	0.5%	Scrubbing the Chamber Floor	90 ml/ sq meter	--	5	1/12	12/12	91.7
						10	0/12	12/12	100.0
						20	0/12	7/12	100.0
						30	0/12	5/12	100.0

Table 4
The Disinfective Effect of Lactic Acid on the Air-borne Influenze Virus

Virus		Chemical Disinfectants					No of Animals Died/Number Infected		
LD_{50} Titer	Calculated Viral Concentration in Air After Spraying	Concentration	Methods of Application	Amount Used	Calculated Viral Concentration in Air After Spraying	Reaction Time, Min.	Experimentals	Controls	Disinfective Efficiency %
$10^{-5.5}$	4.98 LD_{50}/L	1/210	Spraying	0.005 ml/L of air	0.02 mg/L	5	10/12	12/12	16.8
						10	6/12	12/12	50.0
						20	4/12	7/12	42.8
						30	2/12	5/12	60.0
$10^{-6.2}$	25.0 LD_{50}/L	1/84	"	"	0.05 mg/L	5	9/12	12/12	25.0
						10	7/12	12/12	41.6
						20	4/12	9/12	55.6
						30	2/12	6/12	66.6
$10^{-5.6}$	6.32 "	1/42	"	"	0.10 "	5	9/12	12/12	25.0
						10	4/12	11/12	63.0
						20	1/12	6/12	83.4
						30	1/12	4/12	75.0
$10^{-6.2}$	25.0 "	1/21	"	"	0.02 "	5	7/12	12/12	41.6
						10	3/12	12/12	75.0
						20	1/12	9/12	88.8
						30	0/12	6/12	100.0

Table 5
The Disinfective Effect of Formalin on Air-borne Influenza Virus

Virus		Chemical Disinfectants					No of Animals Died/Number Infected		
LD_{50} Titer	Calculated Viral Concentration in Air After Spraying	Concentration	Methods of Application	Amount Used	Calculated Viral Concentration in Air After Spraying	Reaction Time, Min.	Experimentals	Controls	Disinfective Efficiency %
$10^{-5.5}$	4.98 LD_{50}/L	3%	Spraying	0.005 ml/L of air	0.05 mg formaldehyde/L	5	8/12	12/12	33.3
						10	4/12	12/12	66.6
						20	2/12	7/12	71.5
						30	0/12	5/12	100.0
$10^{-6.2}$	25.0 LD_{50}/L	6%	Spraying	0.005 ml/L of air	0.10 mg formaldehyde/L	5	7/12	12/12	41.5
						10	4/12	12/12	66.5
						20	3/12	9/12	66.6
						30	0/12	7/12	100.0
$10^{-5.5}$	4.98 LD_{50}/L	12 %	Spraying	0.005 ml/L of air	0.20 mg formaldehyde/L	5	2/12	12/12	83.2
						10	3/12	12/12	75.0
						20	0/12	6/12	100.0
						30	0/12	5/12	100.0

Table 6
The Disinfective Effect of Ethanol and Glycerol
on the Air-borne Influenza Virus

Virus		Chemical Disinfectants					No of Animals Died/Number of Infected		
LD_{50} Titer	Calculated Viral Concentration in Air After Spraying	Concentration	Methods of Application	Amount Used	Calculated Viral Concentration in Air After Spraying	Reaction Time, Min.	Experimentals	Controls	Disinfective Efficiency %
$10^{-5.5}$	4.98 LD_{50}/L	10% Ethanol	Spraying	0.005 ml/L of Air	0.4 mg/L	5	12/12	12/12	0
						10	12/12	12/12	0
						20	7/12	8/12	12.5
						30	3/12	5/12	40.0
$10^{-5.5}$	4.98 LD_{50}?L	20% Ethanol	Spraying	"	0.8 "	5	12/12	12/12	0
						10	12/12	12/12	0
						20	7/12	8/12	12.5
						30	4/12	5/12	20.0
$10^{-5.6}$	6.32 "	4% Glycerol	Spraying	"	0.2 "	5	12/12	12/12	0
						10	12/12	12/12	0
						20	7/12	6/12	-16.6
						30	5/12	4/12	-25.0
$10^{-5.6}$	6.32 "	8% Glycerol	Spraying	"	0.4 "	5	12/12	12/12	0
						10	12/12	12/12	0
						20	8/12	6/12	-33.3
						30	4/12	4/12	0

Discussion

According to Edward, et al., Vashkov, and Tsui-pu-li-ssu-k'a-ya, hypochlorous acid, calcium or sodium hypochlorite, and bleaching powder solution are highly effective in the disinfection of the air-borne influenza virus. However, Chang, et al., believe that these chemicals are not so reliable, and their experiments have demonstrated that lactic acid is the most effective disinfectant. In our experience, calcium hypochlorite solution has a high disinfective efficiency at a concentration of 0.0034 mg chlorine per liter of air, but it has a much lower efficiency at 0.0017 mg chlorine per liter of air.

Apparently, the content of active chlorine in bleaching powder and calcium hypochlorite has a great deal to do with their disinfective effect. Since Change et al., have never reported the active chlorine content of hypochlorite used in their experiments, it is possible that their chemicals have a very low chlorine content, and therefore a low disinfective effect. Commercial bleaching powder contains only 25% active chlorine, so it is necessary to use it in an amount two to three times greater than that used in this experiment in order to obtain the same disinfective effect.

Our result with lactic acid agrees with that of Chang, et al., except for the fact that lactic acid acts much more slowly than calcium hypochlorite. Furthermore, it is not economical to use lactic acid, since a large quantity of lactic acid has to be used for an effective disinfection.

Holm et al., have made a systematic study on the disinfective effect of formalin aerosol. They utilize the heat evolved from the reaction between formalin and potassium permanganate to vaporize formaldehyde, which , at a concentration of 0.11-0.28 mg per liter of air, exerts a strong disinfective effect on a variety of the laboratory bacteria, though at a rate varying with the humidity of the air.

Base has demonstrated that the concentration of formaldehyde in the air may be lowered from its adsorption to the surfaces of wall, paper, or wood. However, this experiment proves that formaldehyde is a more efficient and reliable disinfectant against influenza as long as a certain concentration of it is maintained in the air. Formaldehyde is a local irritant, but no such injury has been observed at the concentration used in this experiment.

Ethanol and other monohydroxyl alcohols may have some disinfective effect against the influenza virus, but they are not reliable. Besides, they are dangerous if used as an aerosol, since they are inflammable. Pulvertaft has reported that the disinfective effect of some disinfectants is decreased upon the addition of glycerol. Our finding shows that glycerol has no disinfective effect on the air-borne influenza virus. In regard to the apparent increase in the death rate of animals, perhaps it is an experimental error or perhaps glycerol aerosol has a protective effect on the virus.

Hence a definite disinfective effect may be obtained from the use of purified or crude bleaching powder, formalin, or lactic acid during the prevalence of influenza. Since bleaching powder causes discoloration of clothing and corrosion of metals, it cannot be used in the house and factory. However, an application of bleaching powder solution for floor-scrubbing, and formalin or lactic acid for spraying, may be suitable in many situations.

Conclusion

The aerosol of calcium hypochlorite (purified bleaching powder), formalin, and lactic acid have a disinfective effect, at a certain concentration, on the air-borne influenza virus. Among them, calcium hypochlorite is the most effective one, followed in order by formalin and lactic acid. A high disinfective effect can also be obtained by scrubbing the floor of the cloudy chamber with a solution of calcium hypochlorite.

Ethanol and glycerol aerosols have no appreciable effect in the disinfection of air-borne influenza. On the contrary, glycerol appears to increase the death rate of the infected white mice.

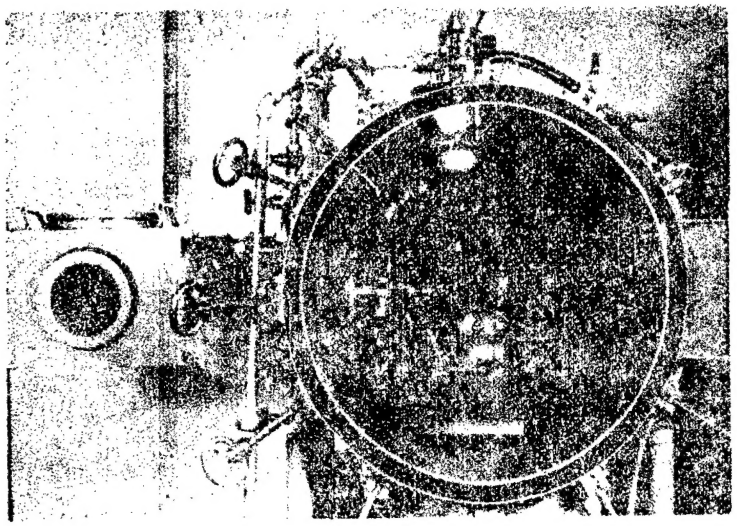


Plate 1

Front View of
Infection Tank

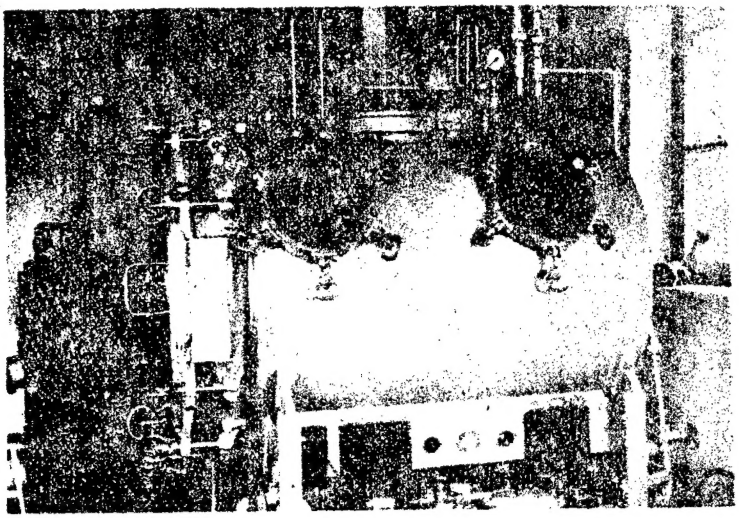


Plate 2

Left Exterior of
Infection Tank

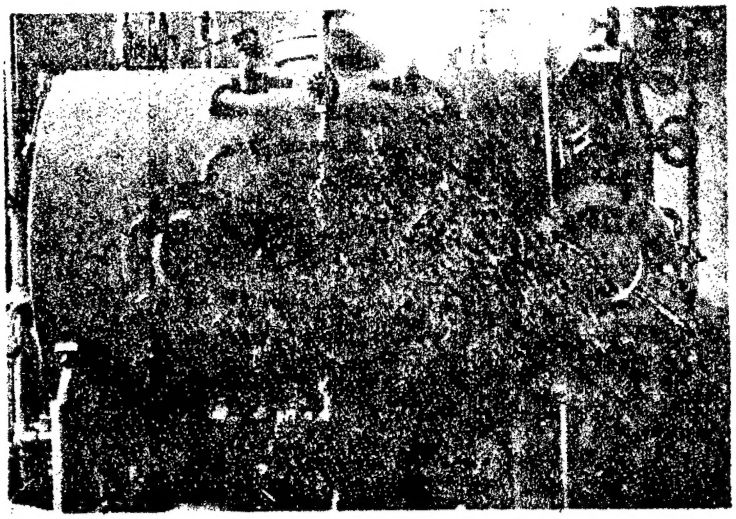


Plate 3

Right Exterior of
Infection Tank

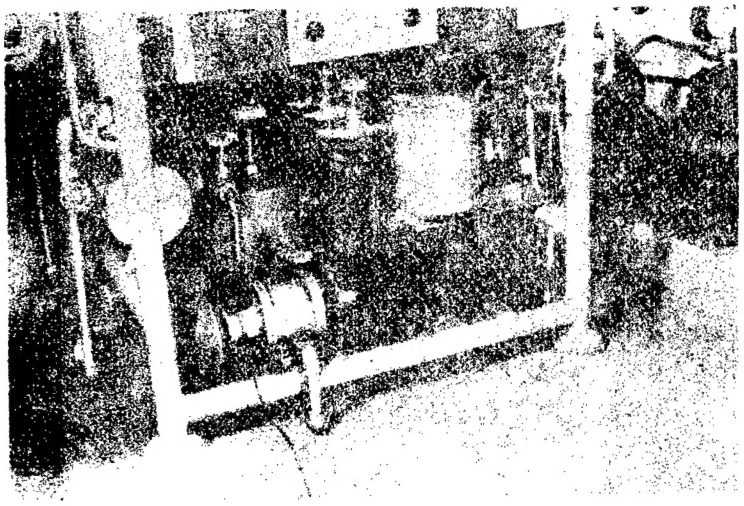


Plate 4

Regulating Devices Installed Below the Infection Tank

Plate 5 The Spray Apparatus

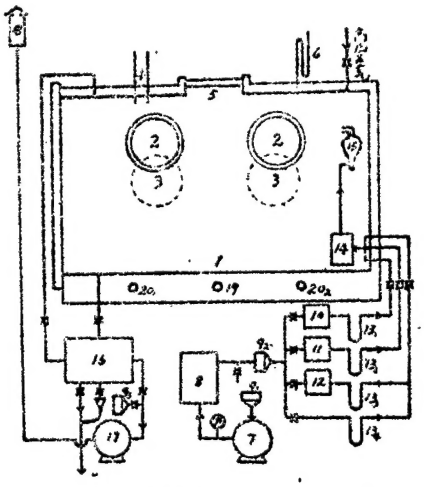


Plate 6

Assembly of Complete Air-Borne Infection Equipment

PLATE DESCRIPTIONS

Plate 1

Photograph shows a close-up front view of an experimental air-borne infection tank with front door open to reveal equipment on the inside such as gloves, spray apparatus, animal cage, etc. Rectangular box-like structure at the left of the photograph is the animal transfer chamber.

Plate 2

Photograph shows two glove ports situated on left side (looking from rear to front end) of cylindrical tank; an observation window, hygrometer, temperature gauge, and pressure gauge on top side; and a control panel at the base.

Plate 3

Photograph shows two round doors which indicate the position of the two air-tight chambers (air tight when the doors are closed). The open door reveals an animal cage on the inside. Samples of inside air are drawn from the two curve-pipe taps installed above these doors.

Plate 4

Photograph shows equipment installed under the control panel. These include an air compressor, airometer, humidity control apparatus, dryer, cooler, air filter, exhauster, etc.

Plate 5

Photograph shows close-up view of spray apparatus mounted on a stand.

Plate 6

Block diagram shows position of components numbered as follows: 1) infection tank, 2) gloves, 3) air-tight chambers, 4) temperature and humidity gauge, 5) observation window, 6) pressure meter, 7) air compressor, 8) cooler, 9_{1-3}) dust separators, 10) dryer, 11) heater, 12) humidity regulator, 13_{1-4}) airometers, 14) gas mixer, 15) spray apparatus, 16) water tank, 17) exhauster, 18) incinerator, 19) temperature regulator with double walls set in sandwich-wise, and 20_{1-2}) heating elements.

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